S.C. Department of Health and Environmental Control

Determination of Total Microcystins and Cylindrospermopsin in Ambient Water

Bureau of Water- Aquatic Science Programs

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1. SCOPE AND APPLICATION

1.1 Method Description

These methods are used for the determination of algal toxins in ambient water, including (extracellular and intracellular) microcystins and cylindrospermopsin via enzyme-linked immunosorbent assay (ELISA). The detection limit for the Microcystin ADDA assay is 0.10 ppb (µg/L) and the detection limit for the Microcystins ADDA SAES assay is 0.016 ppb (µg/L). The detection limit for the Cylindrospermopsin assay is 0.040 ppb (µg/L). The detection limit for using the seawater sample treatment solution for Cylindrospermopsin is 0.015ppb (ug/L).

2. METHOD SUMMARY

The method is an immunoassay for the quantitative and sensitive cogener-independent detection of Microcystins and Nodularins and Cylindrospermopsin in ambient water samples. The testing is completed in a 96-well microtiter plate.

2.1 Microcystins

The test is an indirect competitive ELISA for the cogener-independent detection of Microcystins and Nodularins. It is based on the recognition of Microcystins, Nodularins, and their cogeners by specific antibodies. Microcystins, nodularins, and their cogeners when present in a sample and a Microcystins-protein analogue immobilized on the plate compete for binding sites of antibodies in solution. The plate is then washed and a second antibody-HRP label is added. After a second washing step and addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of Microcystins present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

2.2 Cylindrospermopsin

The test is a direct competitive ELISA for the detection of Cylindospermopsin. It is based on the recognition of Cylindrospermopsin by specific antibodies. Cylindrospermopsin, when present in a sample, and a Cylindrospermopsin-HRP analogue compete for the binding sites of rabbit anti-Cylindrospermopsin antibodies in solution. The anti-Cylindospermopsin antibodies are then bound by a second antibody (goat anti-rabbit) immobilized on the wells of the microtiter plate. After a washing step and addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of Cylindrospermopsin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

3. DEFINITIONS

3.1 Analysis Batch

Standards, samples, and quality control elements are assayed on a single 96-well plate using identical lots of reagents and wells. Each plate by definition is an Analysis Batch, regardless of the number of wells included. Quality control samples must be analyzed in each Analysis Batch at the frequencies prescribed. Each Analysis Batch includes the following elements:

- Calibration Standards
- Quality Controls
- Field samples (ambient water)

3.2 Well Replicates

Within the Analysis Batch, this method requires each calibration standard, field sample, and QC sample to be assayed in two wells. These two wells are called well replicates. Two values are associated with each well replicate: an absorbance measured by the plate reader, and a concentration calculated from this absorbance.

3.3 Use of Well Replicate Absorbance Values

For each set of well replicates, the percent coefficient of variation (%CV) is calculated from the two absorbance values. The %CV of the absorbance values for calibration standards must meet QC criteria. The %CV of the absorbance values for all field and QC samples must meet the limits. Refer to Table 2 for QC criteria.

3.4 Use of Well Replicate Concentrations

For each set of well replicates, the mean is calculated from the two concentration values. The mean concentration must be used for reporting field sample results. The mean must be used in all method calculation and for evaluating results against QC limits.

3.5 Calibration Standards

Solutions of Microcystin and Cylindrospermopsin toxins provided in the ELISA kit or prepared in the laboratory that are appropriate for the measurement range of the ELISA kit.

3.6 Calibration Curve

The calibration points are modelled using a four-parameter logistic function, relating concentration (x-axis) to the measured absorbance in the wells (y-axis). Note the inverse relationship between concentration and response. The zero calibration standard gives the highest absorbance and the highest calibration standard gives the lowest absorbance. Note also that the slope, or sensitivity, of

the ELISA response is greatest in the middle of the curve and tends toward zero slope at extreme low and high concentrations.

3.7 Four-parameter Logistic Equation

$$y = \frac{(a-d)}{1+(\frac{x}{c})^b} + d$$

y= absorbance
x= concentration
a= absorbance at the bottom plateau
b= slope related term at the inflection point
c= concentration at the inflection point= EC_{50}
d= absorbance at the top plateau

The coefficients, a, b, c, and d, are calculated by the data reduction software using regression analysis.

3.8 Quality Control Sample (QCS)

A solution containing microcystin toxins or cylindrospermopsin toxins at a known concentration that is obtained from a source different from the source of calibration standards. The purpose of the QCS is to verify the accuracy of the primary calibrations standards.

4. HEALTH AND SAFETY WARNINGS

4.1 Microcystins

The standard solution in the test kit contain small amounts of Microcystins. The substrate solution contains tetramethylbenzidine (TMB) and the stop solution contains diluted sulfuric acid. Avoid contact of the TMB and stopping solution with skin and mucous membranes. If these reagents come in contact with skin, wash with water.

4.2 Cylindrospermopsin

The standard solutions in the test kit contain small amounts of Cylindrospermopsin. The substrate solution contains tetramethylbenzidine (TMB) and the stop solution contains diluted sulfuric acid. Avoid contact of the TMB and stopping solution with skin and mucous membranes. If these reagents come in contact with skin, wash with water.

4.3 Cylindrospermopsin Seawater Sample Reagent

Irritant to skin and mucous membranes. May cause eye irritation in susceptible persons. The chemical, physical, and toxicological properties of this reagent have not been thoroughly investigated.

4.4 Each laboratory is responsible for maintaining an awareness of OSHA regulations regarding safe handling of any chemicals used in this method. A reference file of Safety Data Sheets should be made available to all personnel involved in the analysis. Handle samples and standards using appropriate personal protective equipment.

5. INTERFERENCES

5.1 Numerous organic and inorganic compounds commonly found in water samples have been tested and found not to interfere with this test. However, due to high variability of compounds that may be found in water samples, test interferences caused by matrix effects cannot be completely excluded.

5.2 Samples containing methanol must be diluted to a concentration <1% methanol to avoid matrix effects.

5.3 Mistakes in handling the test can cause errors. Possible sources for such errors include: inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, and extreme temperatures during the test performance (lower than 10°C or higher than 30°C). The assay procedure should be performed away from direct sunlight.

5.4 To avoid cross contamination between samples, do not reuse plastic syringes for filtering. Thoroughly clean glass containers if they are reused. Do not reuse septa from bottle containing ambient water samples.

5.5 As with any analytical technique, positive results requiring regulatory action should be confirmed by an alternative method.

6. SAMPLE HANDLING, PRESERVATION, AND STORAGE

6.1 Collect samples in 500 mL polyethylene terephthalate glycol (PETG) containers with Polytetrafluoroethylene (PTFE) lined septa lids. Use of other types of plastic collection and/or storage containers may result in adsorptive loss of Microcystins, producing inaccurate (falsely low) results. Ambient water samples do not need to be treated after collection. Freeze samples upon arrival at the laboratory. Samples can be stored in the freezer for up to 2 weeks. When freezing, allow adequate volume for expansion and place the sample container on its side to prevent breakage.

6.2 Place samples on ice immediately. The temperature blank in the cooler must not exceed 10°C during the first 48 hours after collection. A temperature of greater than 10°C is acceptable if transit time is short and the samples do not have sufficient time to chill. In this case, examine the ice packs in the cooler. If they remain frozen, the samples are valid. Based on holding time (see section 6.1), refrigerate or freeze samples upon arrival to the laboratory.

6.3 Samples may be filter and assayed any time after lysing if within 14 days of collection. If not assayed immediately, store lysed samples by freezing in glass

vials with PTFE-faced septa, for example, 1 mL of lysed and filtered sample held in a 4mL vial.

7. INSTRUMENTATION AND EQUIPMENT

7.1 Adda ELISA Test Kits- 96-well Microtiter Plates

- **7.1.1** Microcystins/Nodularins- Abraxis PN 520011
- **7.1.2** Microcystins-ADDA SAES- Abraxs PN 520011SAES
- **7.1.3** Cylindrospermopsin- Abraxis PN 522011
- **7.1.4** Standards
  1. Microcystins ADDA: (6): 0, 0.15, 0.40, 1.0, 2.0. 5.0 ppb, 1mL each
  2. Microcystins ADDA SAES: (6): 0, 0.05, 0.15, 0.4, 1.5, 5.0 ppb, 1mL each
  3. Cylindrospermopsin: (7): 0, 0.05, 0.10, 0.25, 0.50, 1.0, 2.0 ppb, 1mL each
- **7.1.5** Control:
  1. Microcystins: 0.75 ± 0.185 ppb, 1 mL
  2. Cylindrospermopsin: 0.75 ± 0.15ppb, 1 mL
- **7.1.6** Sample Diluent, 25 mL, for use as a Laboratory Reagent Blank and for dilution of samples above the range of the standard curve
- **7.1.7** Antibody Solution
  1. Microcystins ADDA: 6mL
  2. Microcystins ADDA SAES, 6mL
  3. Cylindrospermopsin: rabbit anti-Cylindrospermopsin, 6 mL
- **7.1.8** Conjugate Solution
  1. Microcystins ADDA: Anti-Sheep-HRP conjugate solution, 12 mL
  2. Microcystins-ADDA SAES Conjugate Solution, 12mL
  3. Cylindrospermopsin: Cylindrospermopsin-HRP conjugate solution (vortex before use), 6 mL
- **7.1.9** Wash Buffer (5X) Concentrate, 100 mL, must be diluted prior to use
- **7.1.10** Substrate (Color) Solution (TMB), 12 mL
- **7.1.11** Stop Solution
  1. 6 mL for Microcystins
  2. 12mL for Cylindrospermopsin
- **7.1.12** Cylindrospermopsin Seawater Sample Treatment Solution, 45 test

7.2 Cyanotoxin Manual Assay System- Abraxis PN 475010S. Includes:

- **7.2.1** Microplate Reader, Model 4303
- **7.2.2** Pipette, transfer, 10-100 µL, adjustable
- **7.2.3** Pipette, repeating, manual
- **7.2.4** Pipette, multichannel, 8-tip, adjustable
- **7.2.5** Basin, reagent, for multichannel, 50/bag
- **7.2.6** Rack for 4mL vials, 48-postion (4x12)

7.3 Disposable plastic tips for pipettes

7.3.1 Cartridges, Repeater, 1mL, bx/100- PN 70468

7.3.2 Tips, Pipette, 10-200µL, 96/bx- PN 300002

7.3.3 Tips, Pipette, 30-300µL, 96/bx- PN 300004

7.4 Vials for freezing samples

7.4.1 Vials, Glass, Clear, 4 mL with caps

7.4.2 Vials, Glass, Clear, 40mL with caps

7.5 Syringes and Filters for Lysing

7.5.1 All plastic Luer-Lok syringes, 3mL, from Thermofisher Scientific

7.5.2 Glass Fiber Syringe Filters, 25mm, 1.2µm,

7.6 500 mL PETG containers with PTFE septa lined lids

7.7 Parafilm for plate covering

8. REAGENTS, STANDARDS, AND CONSUMABLE MATERIALS

8.1 Analysis Kit

Store kits according to manufacturer's instructions. Standards and reagents may be used until the manufacturer's expiration date.

8.1.1 Both the Microcystin and Cylindrospermopsin kits should be stored in the refrigerator (4-8°C). The solutions must be allowed to reach room temperature (20-25 °C) before use. Consult state, local, and federal regulations for proper disposal of all reagents.

9. INSTRUMENT CALIBRATION PROCEDURES

9.1 Micropipetters

Micropipetters must be verified each year for accuracy. Verification of accuracy is done by pipetting DI water and then weighing to determine if it is accurate. This check must be done for 50µL, 100 µL, and 250 µL.

9.2 Calibration Procedure

A calibration is required with each Analysis Batch. Use the concentrations stated in the kit instructions. Do not add additional calibration levels or eliminate any levels. Use the calibration standards provided in the original kit. Each calibration standard must be added to at least two wells.

9.3 Calibration Acceptance Criteria

The calibration curve is validated by evaluating the %CV of the absorbance values for the well replicates representing each calibration level, and the correlation coefficient of the four-parameter logistic curve. Calculate the %CV for each of the paired absorbance values, including the "zero" standard. The %CV for each pair must be less than, or equal to, 10%. However, one pair is allowed to exceed 10% providing the %CV is less than, or equal to, 15%. The square of the correlation coefficient (r^2) of the four-parameter curve must be greater than, or equal to, 0.98.

If the calibration fails the %CV limits or r^2 is less than 0.98, then the entire Analysis Batch is invalid. Assay the samples in a subsequent Analysis Batch. Freeze the filtered samples if this Analysis Batch cannot be completed on the same day as the original attempt. Each sample must be within the 14-day holding time for the repeat assay.

10. Procedures

10.1 Sample Lysing Procedure by Freeze-Thaw

10.1.1 Mix samples thoroughly and immediately transfer 5 to 10 mL of each field sample into a 40 mL vial to begin three freeze-thaw cycles. If the sample was previously frozen, only two freeze-thaw cycles are needed (once it has thawed, it has undergone the first freeze/thaw cycle). Smaller vials may be used, but reduce the sample volume to less than 25% of vial capacity.

10.1.2 Once sample is completely frozen, remove from freezer and thaw. To speed up the process, vials may be immersed in a 35°C in a water bath until completely thawed. Ensure samples are completely frozen and completely thawed during each cycle.

10.1.3 Filter 1 to 2 mL of each lysed sample into a 4mL vial using a glass-fiber syringe filter. Samples are ready for immediate analysis.

10.2 Seawater Sample Preparation

10.2.1 Microcystins

1. No matrix effects have been observed with seawater salinities (salinity up to 38 parts per thousand) using the ADDA SAES ELISA plate

10.2.2 Cylindrospermopsin

1. Weigh 0.1 g of Cylindrospermopsin Seawater Sample Treatment reagent into a clean, appropriately labeled 4mL glass vial
2. Add 1mL of brackish water or seawater sample to the vial
3. Vortex for 1 minute. Allow the sample to settle for 10 minutes
4. Pipette the supernatant into an appropriately labeled microcentrifuge tube. Centrifuge for 5 minutes at 13,000 rpm. The sample will separate into 3 laters: a solid, white precipitate (bottom layer), a clear liquid (center layer), and a very thin white film (on top of the liquid layer).
5. Pipette the clear liquid (center layer) into a clean, appropriately labeled 4mL glass vial. Avoid pipetting the very thin white film

6. Dilute the supernatant 1: 3 with DI H2O (I.e. 333 uL supernatnat and 667 ul DI H2O). The sample can then be analyzed using the Abraxis Cylindrospermopsin ELISA Kit.

10.3 Test Preparation

10.3.1 Verify kit standards and reagents are used prior to the expiration date. Allow the reagents and samples to reach ambient temperature before analysis. The assay procedure must be performed away from direct sunlight.

10.3.2 Remove the number of microtiter plate strips required from the resealable pouch. The remaining strips are stored in the pouch with the desiccant (tightly sealed)

10.3.3 The standards, control, sample diluent, antibody enzyme conjugate, substrate, and stop solutions are ready to use and do not require any further dilutions

10.3.4 Dilute the wash buffer (5X) concentrate at a ratio of 1:5 with deionized or distilled water. If using the entire bottle (100mL), add to 400mL of deionized or distilled water and mix thoroughly.

10.3.5 The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously. See Table 1.

10.4 Assay Procedures

10.4.1 Microcystins

1. Add 50µL of the standard solutions, control, or samples into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate is recommended.
2. Add 50µL of the antibody solution to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for 90 minutes at room temperature.
3. Remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. Wash the strips three times using the diluted wash buffer. Please use at least a volume of 250 µL of 1X wash buffer for each well and each washing step. Blot the inverted plate after each wash step on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.

4. Add 100 µL of the enzyme conjugate solution to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strip for 30 minutes at room temperature.
5. Remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. Wash the strip three times using the diluted wash buffer. Please use at least a volume of 250 µL of 1X wash buffer for each well and each washing step. Blot the inverted plate after each wash step on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.
6. Add 100 µL of substrate (color) solution to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for 20-30 minutes at room temperature. Protect the strips from sunlight.
7. Add 50 µL of stop solution to the wells in the same sequence as for the substrate (color) solution using a multi-channel pipette or a stepping pipette.
8. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

10.4.2 Cylindrospermopsin

1. Add 50 µL of the standards, control (QCS), LRB, or samples into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate is recommended.
2. Add 50 µL of the enzyme conjugate solution to the individual wells successively using a multi-channel, stepping, or electronic repeating pipette.
3. Add 50 µL of the antibody solution to the individual wells successively using a multi-channel, stepping, or electronic repeating pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for 45 minutes at room temperature.

4. Remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. Wash the strips four times using the diluted wash buffer. Please use at least a volume of 250 µL of 1X wash buffer for each well and each washing step. Blot the inverted plate after each wash step on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.
5. Add 100 µL of substrate (color) solution to the individual wells successively using a multi-channel, stepping, or electronic repeating pipette. Cover the wells in the same sequence as for the substrate (color) solution using a multi-channel, stepping or electronic repeating pipette.
6. Add 100 µL of stop solution to the wells in the same sequence as for the substrate (color) solution using a multi-channel, stepping, or electronic repeating pipette.
7. Read the absorbance at 450nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

10.5 Running an Assay

10.5.1 Place the plate instrument with well A-1 at the rear right corner so that row 1 is going into the reader first. As you press the first row back and down you will feel slight tension on the plate stretching the carrier so that the front fits in. The plate requires a snug fit.

10.5.2 When using a strip tray, make sure wells are pushed down into tray so that they will not cause the plate to jam or entry. Use care that well tabs do not extend over other wells. Do not place the tabbed ends of strips in row 1; they should be in row 12. Be sure to place the strips in the order in which Blanks, Calibrators and Samples are to be read.

10.5.3 For best results, do not fill wells completely; 200-250 µL depending on well total volume is the maximum fill recommended when the mixing feature is used.

10.5.4 Plate Layout is the default window for Abraxis Reader and displays when the program is started. There are several options: Load Plate, Save Plate, Reset, Re-Assign, Read Plate or Remove. Once samples have been assigned, press the Read Plate button to run. Results are displayed as delta Abs for fixed time read, and delta Abs/min for non-fixed time kinetic. Refer to the "AReader Abraxis Model 4303 Operators Manual" for more information on running an assay.

10.5.5 Sample analyses resulting in a higher concentration than the highest standard in the calibration curve must be diluted within the calibration range and reanalyzed to obtain accurate results. Samples may not be diluted in the well plate. If a sample is diluted, the final values must be

calculated by multiplying the result by the proper dilution factor. Report calculated values.

10.5.6 Save and print a copy of the calibration curve and sample results as part of the laboratory's record maintenance protocol.

10.5.7 Semi-quantitative results can be derived by simple comparison of the sample absorbances to the absorbances of the standards.

10.4.7.1 Samples with lower absorbances than a standard will have concentrations of Microcystins or Cylindrospermopsin greater than the standard. Samples which have higher absorbances than a standard will have concentrations of Microcystins or Cylindrospermopsin less than that standard.

10. 5 QUALITY CONTROL

QC requirements include the IDC, and QC elements associated with each Analysis Batch. This section describes each QC parameter, its required frequency, and the performance criteria that must be met in order to satisfy EPA data quality objectives. These QC requirements are considered the minimum acceptable QC protocol. Laboratories are encouraged to institute additional QC practices to meet their specific needs.

10.5.1 Initial Demonstration of Capability (IDC)

The IDC must be successfully performed prior to analyzing field samples. A plate with all calibration standards, controls, and LRB, plus 10 field samples, must be ran in duplicate wells for the IDC. The IDC must be performed by each analyst, when a new analyst begins work or whenever a change in analytical performance.

When conducting the IDC, the analyst must meet the calibration requirements specified in section 9 for the standards. The %CV for each pair must be less than, or equal to, 10%. However, one pair is allowed to exceed 10% providing the %CV is less than, or equal to, 15%. All samples must have a %CV of less than 15%. If the analyst fails to meet the %CV limits or $r^2 = 0.98$ for the given standards, then their batch is invalid and they must perform the analysis in a subsequent Analysis Batch. The mean recovery of the QCS must also have a percent recovery $\geq$70% and $\leq$130% of the true value. If the analyst fails to meet the percent recovery during the IDC, then the analysis batch is invalid and must be performed again in a subsequent Analysis Batch.

10.5.2. Criterion for Replicate Wells

All field and QC samples are added to at least two wells. The %CV of the absorbance values measured for the well replicates must be less than, or equal to, 15%. Calculate the %CV as follows:

$$\%CV = \frac{Standard\ Deviation\ of\ Absorbances}{Mean\ Absorbance} x100\%$$

If the %CV exceeds 15% for a field sample or QC sample, then that sample is invalid. Note that the well replicates of calibration standards must meet a different set of criteria for %CV.

10.5.3 Quality Control Standard (QCS)

A secondary source QCS must be analyzed with each batch of samples to verify the concentration of the calibration curve. If a QCS is already included in the kit, it may be used if it has a different lot number than the calibration standards and was prepared from a separate primary stock. Acceptance limits must be within ±25% of true value. QCS values exceeding the acceptance limits require action and reanalysis of sample(s) with results greater than the concentration of an acceptable Low-CV in the same analytical batch. If reanalysis is not possible, all sample concentration results greater than an acceptable Low-CV analyzed in the same batch must be appropriately qualified and noted in the final report.

11 DATA REDUCTION, VALIDATION, AND REPORTING

11.1 Quantitation

A four-parameter logistic curve fit must be used. Other curve-fitting models are not permitted. Calculate the sample concentration for each well using the multipoint calibration. For each field and QC sample, average the two concentration values from each well. Use this mean to report sample results and to evaluate QC results against acceptance limits. Final results should be rounded to two significant figures.

11.2Exceeding the Calibration Range

If a result exceeds the range of the calibration curve, dilute the sample with reagent water. Analyze the diluted sample in a subsequent Analysis Batch. Incorporate the dilution factor into the final concentration calculations. Report the dilution factor with the sample result.

12 WASTE MANAGEMENT

The EPA requires that laboratory waste management practices be consistent with all applicable rules and regulations, and that laboratories protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. In addition, compliance is required with any sewage discharge permits and regulations, particularly the hazardous waste identification rules and land disposal restrictions.

13 REFEREENCES

EPA Method 546, "*Determination of Total Microcystins and Nodularins in Drinking Water and Ambient Water by Adda Enzyme-Linked Immunosorbent Assay*; EPA 815-B-16-011; Office of Water: Cincinnati, OH, August 2016.

14 REVISION HISTORY

Revision	Date	Summary	Section
1	03/05/20	Added limit detection for Microcystins ADDA-SAES and for use of Cylindrospermopsin seawater sample treatment	1.1
1	03/05/2020	Added safety information about the Cylindrospermopsin seawater sample treatment	4.3
1	03/05/20	Added limitations with methanol	5.2
1	03/05/20	Changed 1 L PETG container to 500mL	6.1
1	03/05/20	Added Microcystins ADDA-SAES test kit supplies	7.1
1	03/05/20	Added Cylindrospermopsin seawater sample treatment to supplies	7.1.12
1	03/05/20	Changed 1 L PETG container to 500mL	7.6

15 Tables, Figures, and Method Performance Data

Table 1. Working Scheme of microtiter plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Std 0**	**Std 4**	**Sample 2**									
B	**Std 0**	**Std 4**	**Sample 2**									
C	**Std 1**	**Std 5**	**Sample 3**									
D	**Std 1**	**Std 5**	**Sample 3**									
E	**Std 2**	**Control**	**Etc.**									

F	**Std 2**	**Control**	**Etc.**									
G	**Std 3**	**Sample 1**										
H	**Std 3**	**Sample 1**										

** Note: The working scheme of the Cylindrospermopsin plate contains an additional standard. Thus well G2 and H2 will be used for Standard 6 and the samples will start in the wells in column 3.

Table 2. Analysis Batch QC Requirements

Method Reference	Requirement	Specification and Frequency	Acceptance Criteria
9	ELISA Calibration- with provided standards	Use kit-recommended levels and concentrations. Two well replicates per standard.	%CV of absorbance ≤10%; ≤15% allowed for 1 pair. $r^2 \geq 0.98$
3.2	Well Replicates	Assay field and QC samples in two wells	Sample invalid if %CV of absorbance values > 15%
3.11	Quality Control Sample (QCS)	Assay 1 QCS for each new lot of calibration standards. Prepare the QCS near the EC_{50} with MC-LR from a source independent of the calibration standards.	Percent recovery ≥70% and ≤130% of the true value.